A non-persistently transmitted-virus induces a pull–push strategy in its aphid vector to optimize transmission and spread

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A B S T R A C T

Plant viruses are known to modify the behaviour of their insect vectors, both directly and indirectly, generally adapting to each type of virus–vector relationship in a way that enhances transmission efficiency. Here, we report results of three different studies showing how a virus transmitted in a non-persistent (NP) manner (*Cucumber mosaic virus*; CMV, *Cucumovirus*) can induce changes in its host plant, cucumber (*Cucumis sativus* cv. Marumba) that modifies the behaviour of its aphid vector (*Aphis gossypii* Glover; Hemiptera: Aphididae) in a way that enhances virus transmission and spread non-viruliferous aphids changed their alighting, settling and probing behaviour activities over time when exposed to CMV-infected and mock-inoculated cucumber plants. Aphids exhibited no preference to migrate from CMV-infected to mock-inoculated plants at short time intervals (1, 10 and 30 min after release), but showed a clear shift in preference to migrate from CMV-infected to mock-inoculated plants 60 min after release. Our free-choice preference assays showed that *A. gossypii* alates preferred CMV-infected over mock-inoculated plants at an early stage (30 min), but this behaviour was reverted at a later stage and aphids preferred to settle and reproduce on mock-inoculated plants. The electrical penetration graph (EPG) technique revealed a sharp change in aphid probing behaviour over time when exposed to CMV-infected plants. At the beginning (first 15 min) aphid vectors dramatically increased the number of short superficial probes and intracellular punctures when exposed to CMV-infected plants. At a later stage (second hour of recording) aphids diminished their feeding on CMV-infected plants as indicated by much less time spent in phloem salivation and ingestion (E1 and E2). This particular probing behaviour including an early increase in the number of short superficial probes and intracellular punctures followed by a phloem feeding deterrence is known to enhance the transmission efficiency of viruses transmitted in a NP manner. We conclude that CMV induces specific changes in a plant host that modify the alighting, settling and probing behaviour of its main vector *A. gossypii*, leading to optimum transmission and spread of the virus. Our findings should be considered when modelling the spread of viruses transmitted in a NP manner.

1. Introduction

Plant viruses as obligate parasites, need to move from host to host to survive. Although other virus transmission ways are possible, vectors transmit most of the known plant viruses. Insects, particularly hemipterans with piercing-sucking mouthparts (aphids, whiteflies and leafhoppers mainly), are by far the most frequent and efficient vectors of plant viruses (Nault, 1997; Hogenhout et al., 2008). For this reason the knowledge on insect behaviour and dispersal is of key importance to understand virus epidemiology. The behaviour of insect vectors can be altered by vector-borne-viruses such that the frequency and nature of the virus–vector interaction is modified to enhance virus transmission and spread (Eigenbrode et al., 2002; Bosque-Pérez and Eigenbrode, 2011; Mauck et al., 2012; Shrestha et al., 2012; van Molken et al., 2012; Moreno-Delafuente et al., 2013; Huot et al., 2013).

Recently, the "Vector Manipulation Hypothesis" (VMH) has been proposed to explain the strategies plant pathogens use to enhance their own spread by altering the behaviour of their insect vectors (Ingwell et al., 2012). According to the VMH, plant pathogens can influence the behaviour and fitness of their insect vectors in two different ways: directly (mediated by the presence of the virus in the vector's body) and indirectly (mediated by changes occurring in the plant as a consequence of infection). It is expected that most virus-induced changes in plants have positive (or neutral) effects on transmission by vectors and that viruses showing similar virus–vector relationships share similar effects on vector behaviour in a way that transmission efficiency is optimized (Mauck et al., 2012).

Aphid behaviour and virus–vector interactions determine how the virus is transmitted as well as the efficiency of a given aphid species to transmit the virus (Gray and Banerjee, 1999). Thus, persistently-transmitted viruses (PT) (and some semipersistent viruses – SP) are usually transmitted by colonizing aphid species, which need to reach and feed from the phloem to acquire and inoculate de virus effectively. PT viruses have a very specific relationship with their vectors representing a narrow range of species able to transmit them (Gildow and Gray, 1993; Gray and Gildow, 2003). On the other hand, viruses transmitted in a non-persistent (NP) manner, which represent the majority of aphid-borne transmitted viruses, are vectored by many non-colonising aphid species during brief intracellular stylet punctures in superficial plant tissues. Long feeding probes are known to reduce their transmission efficiency (Ng and Falk, 2006). The relationship of viruses transmitted in a NP manner with their aphid vectors is not as specific and intimate as for PT viruses and interactions in these pathosystems are likely limited to indirect effects through the host plant (Nault, 1997; Mauck et al., 2010).

PT virus-infected plants tend to be more attractive and/or arrestant to aphid vectors than healthy ones (Castle et al., 1998; Eigenbrode et al., 2002; Jimenez-Martínez et al., 2004a; Srinivasan et al., 2006; Medina-Ortega et al., 2009; Bosque-Pérez and Eigenbrode, 2011). Moreover, vectors feeding on PT virus-infected plants often have greater nymphal survival, adult fecundity, longevity and/or increased growth rate (Fereres et al., 1989; Castle and Berger, 1993; Jiménez-Martínez et al., 2004b; Srinivasan and Alvarez, 2008).

For NP viruses, studies describing vector attraction and feeding preferences and/or fitness on infected plants are more limited. Recent work by Mauck et al. (2010) with *Cucumber mosaic virus* (CMV, *Bromoviridae*: *Cucumovirus*) showed that winged and wingless morphs of *Aphis gossypii* (Glover) and *Myzus persicae* (Sulzer) are initially attracted by the volatile organic compounds emitted by CMV infected squash plants. However, some time after landing they prefer non-infected plants. Furthermore Eckel (1990), showed that tobacco plants infected with *Tobacco etch virus* (TEV, *Potyviridae*: *Potyvirus*) were more attractive to alighting aphids than non-infected plants. Furthermore, studies conducted with *Soybean mosaic virus* (SMV, *Potyviridae*: *Potyvirus*) showed that *Rhopalosiphum maidis* (Fitch) remained longer on non-infected than on SMV-infected soybean plants before taking off, although *M. persicae* exhibited no preference (Fereres et al., 1999).

The insect's choice to colonize a plant is a complex process involving different stimuli and responses. To find and identify feeding sites when searching for their host plants, phloem-feeding insects follow a series of events that culminate in sustainable phloem sap ingestion if plants are recognized as acceptable (Powell et al., 2006). During their pre-alighting phase, aphids are guided by visual and olfactory cues coming from plants that may act as potentially acceptable hosts. It seems that most aphids species are specially attracted to yellow or yellow-green, which is thought to indicate a favourable nutritional status in terms of soluble nitrogen (Kennedy et al., 1961; Moericke, 1969; Fereres et al., 1999; Döring et al., 2009). Virus infection may produce similar changes in canopy colour as well as in plant volatile compounds emission that favours aphid attraction and landing on the crop (Ajayi and Dewar, 1983; Eigenbrode et al., 2002). That is the case of some NP viruses that promote alate aphid vector attraction to the chlorotic symptoms and the odour emissions emitted by infected plants (Macias and Mink, 1969; Mauck et al., 2010). This attraction of aphid vectors to infected plants may have important consequences on virus spread as models on NP virus transmission show that the number of plants visited per day is a key variable driving virus epidemics (Madden et al., 1990, Madden et al., 2000).

Once aphids land on a plant they make consecutive superficial probes and use gustatory cues to discriminate between host and non-host plants by means of consecutive intracellular stylet punctures. Then, stylets penetrate deeper through the intercellular spaces to reach the vascular bundle and penetrate the phloem sieve elements where they remain feeding for long periods of passive sap ingestion (Fereres and Moreno, 2009). All the above mentioned stylet penetration behaviours can be monitored using the Electrical Penetration Graph (EPG) technique, which records signal waveforms reflecting different insect activities (Tjallingii, 1988). Using EPGs, it has been possible to study the host selection process of pierce-sucking insects as well as the characterization of insect stylet activities associated to the transmission of plant viruses. It is well known that intracellular stylet punctures visualized as potential drops (pd) during brief probes in epidermal and mesophyll cells are responsible of the acquisition and inoculation of NP-viruses (Powell et al., 1995; Martín et al., 1997; Powell, 2005). The number of short probes and number of intracellular stylet punctures (pd) have a positive correlation with the acquisition and subsequent transmission of NP viruses (Collar et al., 1997; Collar and Fereres, 1998). In other words, aphids that make a larger number of brief superficial probes and intracellular stylet punctures transmit NP-viruses with higher efficiency.

Here, we describe a series of time-controlled preference and probing behaviour studies using CMV infected cucumber plants and the aphid vector, *A. gossypii*. A combination of free-choice preference assays conducted in test arenas under semi-field conditions and the EPG technique allowed us to evaluate the indirect effects of CMV infection on the alighting, settling and probing behaviour of non-viruliferous aphids on cucumber (*Cucumis sativus* cv. Marumba) plants. Our results indicate that aphid response to CMV-infected plants at different time intervals have significant implications in the transmission, spread and epidemiology of viruses transmitted in a NP manner and should be considered to construct or tune up existing simulation models and patterns of spread that describe virus disease epidemics.

2. Materials and methods

2.1. Biological material: aphid colonies, plants and virus isolates

A single virginiparous aptarae collected from melon in Almería, Spain, in 1998 was used to initiate a virus-free laboratory culture of *A. gossypii*. This colony was reared on melon plants (*Cucumis melo* L. cv. Primal) for several generations in rearing cages in environmental growth chambers at a 23:18 °C temperature (D:N), photoperiod of 16:8 h (L:D) and 60–80% RH. For each experiment, newly emerged alates (24–72 h after last moult) were collected with an aspirator from the top of the rearing cages on the same day and time in which the experiments were started. We assumed that alate aphids at the top of the cage had terminated their migratory flight phase. Cucumber plants were germinated in 12 cm diameter pots using a mixture of equal parts of vermiculite (No. 3, Asfaltex S.A., Barcelona, Spain) and soil substrate (Kekilla Iberica, Almería, Spain). They were watered three times a week and a nutritional complex of 20-20-20 (N:P:K) Nutrichem 60 fertilizer (Miller Chemical & Fertilizer Corp., PE, USA) was added to the irrigation water in a proportion of 0.25 g/l dosage. Cucumber plants were mechanically inoculated with CMV (isolate M06) (Diaz et al., 2003) obtained from a melon crop in 1996 in Tarragona Spain, and kindly provided by Dr. E. Moriones (EELM-CSIC, Spain) Plants were inoculated 2 weeks after sowing at the 1-true leaf stage and used 4 weeks post-inoculation as viral sources (6-leaf stage). Mock-inoculated cucumber plants (rubbed only with buffer solution) of the same growth stage were used as non-infected controls. All plants were

grown in an insect-proof chamber at 24:20 °C temperature (D:N), a photoperiod of 16:8 h (L:D) and 60–80% RH.

2.2. Alighting and settling behaviour of A. gossypii on CMV-infected plants

Two different semi-field experimental set-ups were carried out in greenhouse facilities at ICA-CSIC to assess the preference of *A. gossypii* for CMV-infected cucumber plants at serial time intervals. Temperature, relative humidity and shadow covers were remotely controlled in the greenhouse through a central computer to ensure the following environmental conditions: temperature of 25:20 °C (D:N), a photoperiod of 16:8 h (L:D) and 75–80% RH.

2.2.1. Dual-choice alighting behaviour assays

The preference of *A. gossypii* alates to alight and remain on CMV-infected and mock-inoculated cucumber plants was assessed at different time periods after aphid release. One CMV-infected and one mock-inoculated cucumber plant were placed 20 cm apart opposite to each other inside an aphid-proof cage (65 cm × 45 cm × 50 cm). The cage structure was made out of wood with organdy on the sides and glass on the ceiling to allow natural light penetrate the cage. Thirty *A. gossypii* alates were released at a time on the youngest expanded leaf of either a CMV-infected or a mock-inoculated plant, according to the treatment. The number of alates remaining on either the CMV-infected or the mock-inoculated plant was counted at different time intervals (1, 10, 30, 60, 120, and 180 min). Each dual-choice assay was replicated 16 times for each treatment and evaluation period.

2.2.2. Aphid settlement behaviour bioassays

The preference of *A. gossypii* to settle on either CMV-infected or mock-inoculated cucumber plants was tested at different time intervals under free choice conditions. We used an arena test similar to the one described by Garzo et al. (2003): six CMV-infected and six mock-inoculated cucumber plants were alternated in a circle inside 1 m × 1 m × 1 m cages equipped with aphid-proof mesh nets. Winged aphids were collected with an aspirator from the rearing colony and grouped in falcon tubes just before the experiments started. Two hundred non-viruliferous winged aphids were placed in a flight platform similar to the one described by Fereres et al. (1999) and released 0.5 m above the test plants. The number of aphids (alates and newborn nymphs) settled on each test plant was counted at short (0.5, 2 and 4 h) and long term (48 h) intervals. For this purpose, each plant was covered by a plexiglass cylinder and transferred to the laboratory for aphid counting. Three replicates per treatment and evaluation period were performed.

2.3. Electrical monitoring of aphid probing and feeding behaviour

The EPG technique was used to monitor plant penetration activities by apterous adult *A. gossypii* on CMV-infected and mock-inoculated cucumber plants. Apterous adult aphids 7–9 days old were immobilized individually using a vacuum-operated plate (Eyela Aspirator A3S, Todyo Rikakikai Co. Ltd., Japan). Then, a thin gold wire (20 μm of diameter) was attached to the dorsum of the aphid with a small droplet of silver glue (16034 Pelco Collodial Silver, Ted Pella Inc., Redding CA, USA). The opposite end of the gold wire was attached to a copper electrode (3 cm long – 1 mm of diameter), representing the input electrode. The output electrode was a copper post (10 cm long – 2 mm of diameter), which was inserted into the plant pot. Aphids with the attached gold wire were placed on the abaxial side of the last expanded leaf of a 6-leaf stage cucumber plant and connected to the DC-EPG device (Giga-4; EPG Systems, Wageningen, The Netherlands). The EPG acquisition procedure was performed inside a Faraday cage to prevent electrical noises. EPG data acquisition was conducted using Stylet+ software for Windows (EPG Systems, Wageningen, The Netherlands). EPG signals were recorded for 8 h under laboratory conditions (22–24 °C) and started immediately after aphids were placed on the cucumber leaf. EPG waveforms previously described for aphids (Tjallingii, 1988) were identified as follows: non-probing (np), intercellular apoplastic stylet pathway (C), intracellular stylet puncture (pd), salivation into phloem sieve elements at the beginning of the phloem phase (E1), passive phloem sap uptake from the phloem sieve elements (E2), active intake of xylem sap (water + nutrients) from xylem elements (G) and mechanical work associated to stylet penetration difficulties (F).

Twenty-eight to thirty replicates per treatment (CMV-infected and mock-inoculated cucumber plants) were recorded. Each replicate (individual aphids and plants) was conducted using a different plant and aphid for each EPG recording. All behavioural variables were processed using the MS Excel Workbook for automatic EPG data calculation, developed by Sarria et al. (2009). Data analysis was conducted for relevant EPG variables for the 8 h of recording period. Furthermore, the data analysis was segmented to understand the evolution of aphid response over time when exposed to CMV-infected and mock-inoculated plants. EPG data was analyzed at the following time intervals: first 15 min, 15–30 min, 0–30 min, 0–60 min (1st hour of recording), 60–120 min (2nd hour of recording), 120–180 min (3rd hour of recoding), and every hour until the eighth hour of recording. Selected EPG variables (mean ± SE) (Table 1) were calculated and compared between treatments as described in Backus et al. (2007): PPW, proportion of individuals that produced a specific waveform type; NWEI, number of waveform events per insect, that is the sum of the number of events of a particular waveform divided by the total number of insects under each treatment; WDI, waveform duration (min) per insect, that is the sum of durations of each event of a particular waveform made by each individual insect that produced that waveform divided by the number of insects that performed that particular waveform under each treatment; and WDE, waveform duration (min) per event, that is the sum of the duration of the events for a

Table 1
Sequential and non-sequential EPG variables used to compare the probing and feeding behavior of *A. gossypii* on CMV-infected and mock-inoculated cucumber plants.

Non-sequential variables
Number of np
Number of probes
Number of pd
Number of C
Number of E1
Number of E2
Total duration of np
Total probing time
Total duration of pd
Total duration of C
Total duration of E1
Total duration of E2
Total duration of E
Sequential variables
Time to 1st probe from start of EPG
Time from start of EPG to 1st E
Time from 1st probe to 1st E
Time from the beginning of that probe to 1st E
Time to from start of EPG 1st sustained E2 (10 min)
Time from 1st probe to 1st sustained E2 (10 min)
Time from the beginning of that probe to 1st sustained E2 (10 min)
Time from the beginning of that probe to 1st E2
Duration of np just after the probe of the first sustained E2
Number of probes to the 1st E1
Number of probes after 1st E
Time from the beginning of the 1st probe to first pd
Time from the end of the last pd to the end of the probe

particular waveform divided by the total number of events of that particular waveform under each treatment.

2.4. Statistical analysis

Statistical analysis of the data was conducted using SPSS 19.0 software package (SPSS, 2010). For the dual-choice alighting behaviour assays the percentage of aphids remaining on each treatment (either CMV-infected or mock-inoculated cucumber plants) was compared by a one-way ANOVA. Data were transformed by arcsin$\sqrt{x}$ before analysis. For the free-choice settling behaviour assays the mean number of winged aphids settling on CMV-infected and mock-inoculated test plants was compared by a *t*-Student test (for Gaussian variables) or Mann–Whitney *U* test (for non-Gaussian variables). Data were transformed by ln $(x+1)$ and checked for normality before analysis using the Shapiro–Wilk *W* test.

All the behavioural variables obtained by EPG recording were transformed prior to analysis by either sqrt $(x+1)$ or ln $(x+1)$ and checked for normality using Shapiro–Wilk *W* test. Comparisons between treatments were made by a Student *t*-test (Gaussian variables) or by Mann–Whitney *U* test (for non-Gaussian variables).

3. Results

3.1. Alighting and settling behaviour of *A. gossypii* on CMV-infected plants

Dual-choice alighting behaviour tests showed no significant differences ($P>0.05$) on aphid preference for CMV-infected or mock-inoculated plants at short time intervals (1, 10 and 30 min after release). However, there was a clear shift in aphid behaviour at 60 min at which point more aphids migrated from CMV-infected to mock-inoculated plants than in the opposite direction, resulting in significantly more aphids found on mock-inoculated than on CMV-infected plants ($F=7.125$, $P=0.012$; Fig. 1). The same trend for alate aphids to alight and remain on mock-inoculated plants rather than CMV-infected plants was observed for the rest of the serial time counts ($F=5.908$, $P=0.021$ for 120 min and $F=5.288$, $P=0.028$ for 180 min).

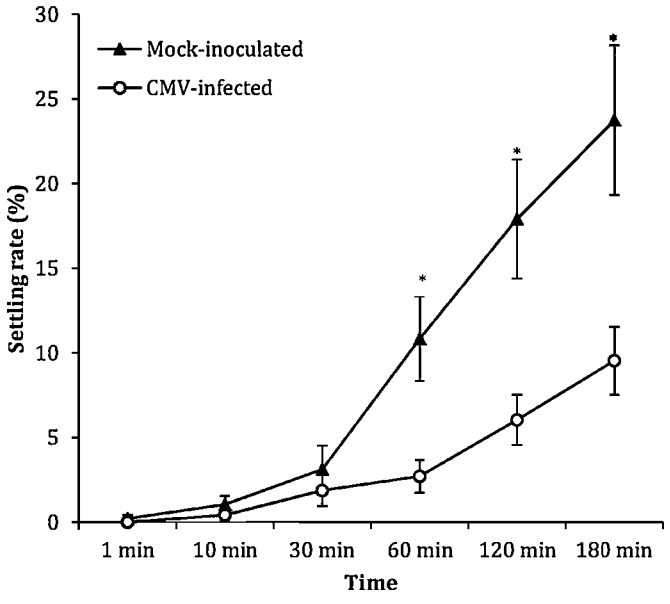

Fig. 1. Effects of CMV-infected plants on the alighting behaviour of *A. gossypii* under dual choice tests. Alighting (%) of *A. gossypii* on a healthy cucumber plant when migrating from a CMV-infected to a mock-inoculated plant and vice versa 1, 10, 60, 120 and 180 min after aphid release. Lines represent the alighting percentage of *A. gossypii* on a mock-inoculated cucumber plant when migrating from a CMV-infected plant (black triangle) and vice versa (white circle) 1, 10, 30, 60, 120 and 180 min after aphid release. *Significant difference ($P<0.05$) according to a one-way ANOVA ($df=1, 30$). Data were transformed by arcsin$\sqrt{x}$ before analysis.

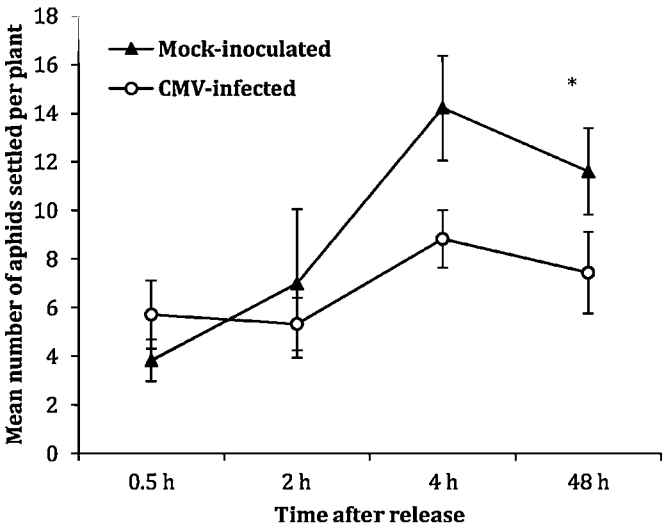

Fig. 2. Mean + SE number of winged *Aphis gossypii* present on CMV-infected and mock-inoculated cucumber plants under free-choice assays at 0.5, 2, 4 and 48 h after aphid release. *Significant differences according to *t*-Student test ($P<0.05$). Data were transformed by ln $(x+1)$ before analysis.

Aphid settling behaviour experiments in the free-choice arena showed that winged *A. gossypii* tend to prefer CMV-infected than mock-inoculated plants at an early stage (0.5 h), although no significant differences between treatments were observed ($F=1.123$, $P=0.213$) (Fig. 2). However, this trend was reversed in the course of time since more aphids settled on mock-inoculated than on CMV-infected plants 2 and 4 h after release from the flight platform, although differences were not statistically significant ($F=0.213$, $P=0.764$ for counts at 2 h and $F=3.299$, $P=0.092$ for counts at 4 h). The mean number of alates was significantly higher ($F=1.303$, $P=0.029$) on mock-inoculated than on CMV-infected plants 48 h after aphid release from the flight platform.

The total number of nymphs on each test plant (Fig. 3) followed the same trend as the total number of alates, with higher counts on mock-inoculated than on CMV-infected plants after 2 and

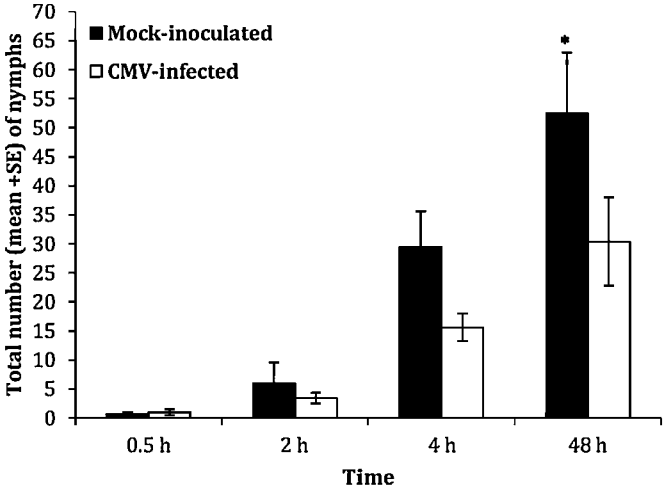

Fig. 3. Total mean number of *A. gossypii* nymphs present on CMV-infected and mock-inoculated cucumber plants at 0.5, 2, 4 and 48 h after the release of winged adults under free-choice conditions. *Significant differences according to *t*-Student test (for Gaussian variables) or Mann–Whitney *U* test (for non-Gaussian variables) ($P<0.05$).

4 h, although differences were not significant ($U = 155.00$, $P = 0.820$ for 2 h and $F = 2.730$, $P = 0.276$ for 4 h). The total mean number of nymphs 48 h after release of winged adults was higher on mock-inoculated (52.5 + 10.5) than on CMV-infected (30.4 + 7.6) plants ($F = 1.688$, $P = 0.029$).

Results of both preference tests (dual-choice alighting and free-choice settling assays) suggest that aphids are attracted in the first 30 min to CMV-infected plants where they start to probe and settle. However, this trend is reverted at some point between the 30–60 min time interval when aphids show no preference for CMV-infected plants and prefer to take off, alight and settle on mock-inoculated plants where they actually remain feeding and reproducing for at least 48 h (Figs. 2 and 3).

3.2. Electrical monitoring of the probing and feeding behaviour of *Aphis gossypii* on CMV-infected plants

The sequential and non-sequential variable values obtained for the stylet penetration activities of *A. gossypii* on CMV-infected and mock-inoculated cucumber plants obtained in the 8 h of EPG recording (0–480 min) did not show any relevant differences between treatments except that a higher proportion of aphids remained in phloem sap ingestion events (E2) on the mock-inoculated than in CMV-infected plants (data not shown).

3.2.1. Short time interval analysis

When EPG variables were analyzed at short time intervals significant differences between treatments were obtained at the 0–15 min but no differences in the 15–30 min time interval frame were recorded (Table 2). Data analysis in the first 15 min showed that aphids made more probes and more intracellular stylet punctures or potential drops "pd" on CMV-infected than on mock-inoculated plants ($P < 0.05$; Table 2). Furthermore, the duration of probes was shorter on CMV-infected than on mock-inoculated plants in the first 15 min of recording. Also, the 1st probe was much shorter on CMV-infected plants and the total duration of intracellular punctures per insect (WDI) was much longer on CMV-infected than on mock-inoculated plants. Significant differences were observed in the time elapsed from the end of the last "pd" to the end of the probe. Such time interval was much lower for aphids probing on CMV-infected than on mock-inoculated plants (Table 2). Conversely, there were no significant differences in the mean duration of intracellular punctures but probes were significantly shorter on CMV-infected than on mock-inoculated plants (WDE).

3.2.2. Analysis of 1 h-frame time intervals

When the EPG variables were analyzed at specific time intervals (1st and 2nd hour of recording), significant differences between treatments were observed (Fig. 4). Data analysis showed that in the 1st hour (0–60 min after the start of EPG recording) aphids made a significantly larger number of probes on CMV-infected than on mock-inoculated plants (Fig. 4a) (NWEI 0–60 min: 9.25 ± 0.79 and 6.07 ± 0.69 for CMV-infected and mock-inoculated plants, respectively [$F = .496$, $P = 0.002$]). The number of intercellular pathway phases (C) was also higher on CMV-infected than on mock-inoculated plants (NWEI 0–60 min: 9.39 ± 0.82 and 6.23 ± 0.71 for CMV-infected and mock-inoculated plants, respectively [$F = 0.657$, $P = 0.003$]) (Fig. 4a). Moreover, the number of times that aphids went back into a non-probe (np) activity (Fig. 4a) was significantly higher for aphids feeding on CMV infected plants (NWEI 0–60 min: 9.60 ± 0.82 and 6.33 ± 0.65 and for CMV-infected and mock-inoculated plants, respectively [$F = 0.593$, $P = 0.002$]). According to the analysis of the 1st hour of recording the mean duration of aphid probes was significantly longer on mock-inoculated than on CMV-infected plants (Fig. 4b) (WDE 0–60 min: 4.99 ± 0.61 and 3.16 ± 0.38 min for mock-inoculated and CMV-infected plants, respectively [$F = 7.248$, $P = 0.007$]). The same result was obtained when the duration of the C waveform (Fig. 4b) was analyzed (WDE 0–60 min: 3.03 ± 0.33 and 1.93 ± 0.20 min for mock-inoculated and CMV infected plants, respectively [$U = 17{,}689$, $P = 0.000$]).

The analysis of the 2nd hour (60–120 min) of recording showed that the durations of the probing and non-probing events (Fig. 4b) remained longer on mock-inoculated than on CMV-infected plants (WDE 60–120 min for non-probe: 4.82 ± 0.58 and 2.93 ± 0.41 min for mock-inoculated and CMV-infected plants, respectively [$U = 8272.00$, $P = 0.007$]; WDE 60–120 min for probe:

Table 2
Sequential and non-sequential EPG variable values (mean ± SE) describing the probing behavior of *Aphis gossypii* Glover on CMV-infected and mock-inoculated cucumber plants at short time intervals (0–15 and 15–30 min).

	Plant	0–15 min			15–30 min		
		PPW	Mean ± SE	P^a	PPW	Mean ± SE	P^a
Number of waveform events per insect (NWEI)							
Probes	Mock-inoculated	30/30	2.10 ± 0.30 a	0.000	30/30	2.17 ± 0.28 a	0.254
	CMV-infected	28/28	4.18 ± 0.44 b		28/28	2.50 ± 0.25 a	
pd	Mock-inoculated	30/30	2.93 ± 0.47 a	0.007	30/30	5.53 ± 1.09 a	0.567
	CMV-infected	28/28	5.64 ± 0.79 b		28/28	4.14 ± 0.80 a	
Waveform duration per insect (WDI)							
1st probe	Mock-inoculated	27/30	169.63 ± 43.75 a	0.000		–	–
	CMV-infected	28/28	39.29 ± 5.38 b				
pd	Mock-inoculated	27/30	25.03 ± 3.03 a	0.006	26/30	35.26 ± 5.28 a	0.200
	CMV-infected	28/28	43.41 ± 5.16 b		25/28	26.27 ± 3.99 a	
Time from the beginning of the 1st probe to 1st pd	Mock-inoculated	27/30	66.46 ± 21.30 a	0.019		–	–
	CMV-infected	28/28	44.79 ± 17.97 b				
Time from the end of the last pd to the end of the probe	Mock-inoculated	27/30	86.32 ± 32.77 a	0.009	26/30	175.65 ± 42.71 a	0.418
	CMV-infected	27/28	33.08 ± 24.55 b		25/28	102.41 ± 35.18 a	
Waveform duration per event (WDE)							
Probes	Mock-inoculated	–	126.79 ± 21.15 a	0.000	–	213.01 ± 30.51 a	0.417
	CMV-infected	–	87.23 ± 13.26 b		–	184.20 ± 27.97 a	
pd	Mock-inoculated	–	7.68 ± 0.40 a	0.875	–	5.52 ± 0.19 a	0.867
	CMV-infected	–	7.69 ± 0.33 a		–	5.66 ± 0.28 a	

PPW, proportion of individuals that produced the waveform type; NWEI, number of waveform events per insect; WDI, waveform duration (seconds) per insect; WDE, waveform duration (seconds) per event (Backus et al., 2007). Probe, probe activity. Waveforms: pd, short intracellular punctures.

[a] Statistical comparisons between the two treatments for each parameter were made by nonparametric Mann–Whitney U test.

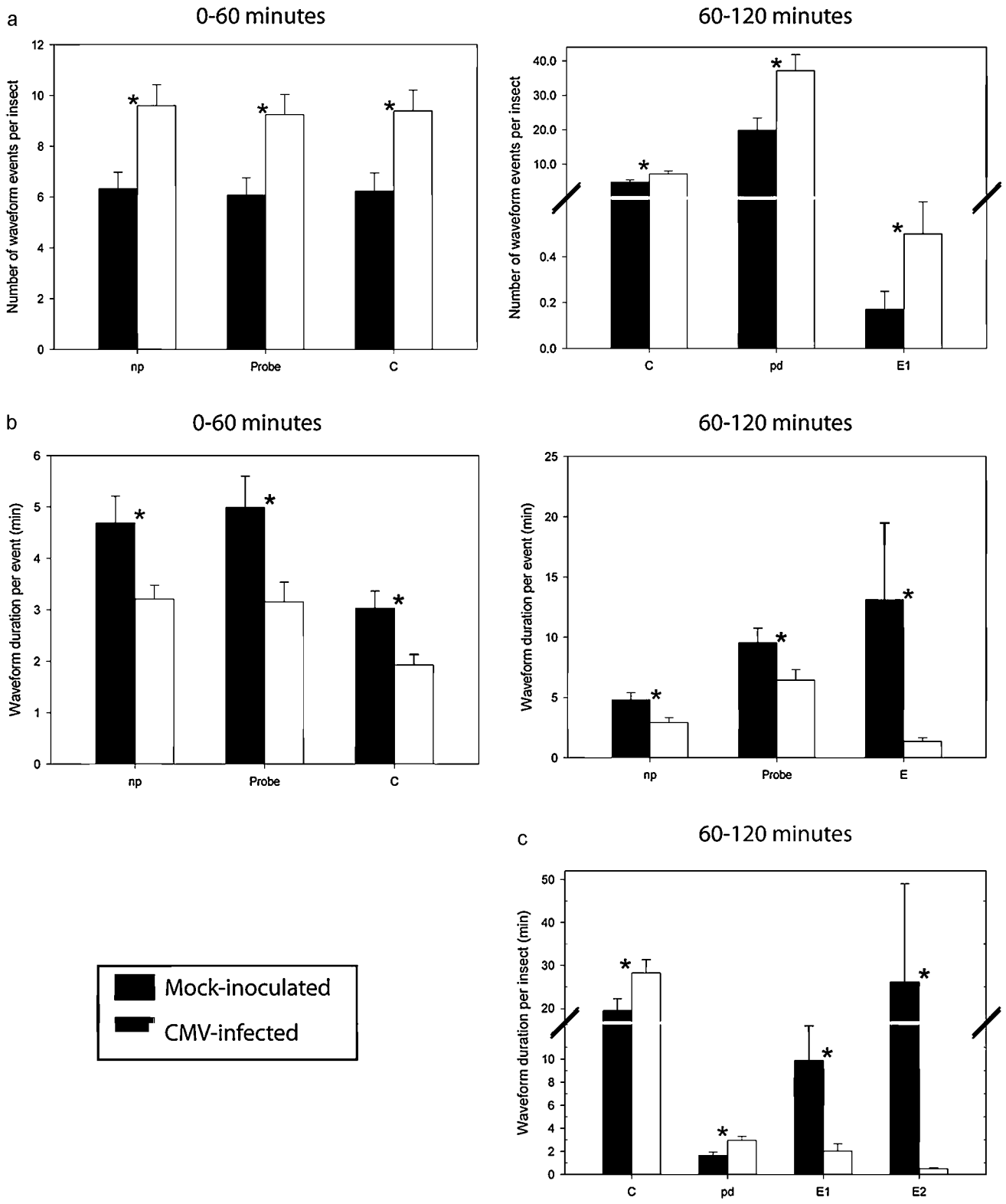

Fig. 4. Analysis of probing and feeding behaviour EPG variables of *Aphis gossypii* on CMV-infected and mock-inoculated cucumber plants in the 1st (0–60 min) and 2nd (60–120 min) hour of recording. (a) Number of waveform events per insect (NWEI); (b) waveform duration per event (WDE); (c) Waveform duration per insect (WDI). *Significant differences according to *t*-Student test (for Gaussian variables) or Mann–Whitney *U* test (for non-Gaussian variables) ($P < 0.05$). Only variables with significant differences between treatments are mentioned in the text.

9.54 ± 1.21 and 6.46 ± 0.84 min for mock-inoculated and CMV-infected plants, respectively [$F = 0.765$, $P = 0.001$]).

We also found that the number of intracellular punctures or "pd" (Fig. 4a) was significantly higher for aphids probing on CMV-infected than on mock-inoculated plants (NWEI 60–120 min: 19.90 ± 3.52 and 37.11 ± 4.71 for mock-inoculated and CMV-infected plants, respectively [$F = 0.373$, $P = 0.005$]) and the mean duration of these "pd" events per insect (Fig. 4c) was longer on

CMV-infected than on mock-inoculated plants (WDI 60–120 min: 1.66 ± 0.28 and 2.95 ± 0.36 min for mock-inoculated and CMV-infected plants, respectively [$U = 241.00$, $P = 0.013$]).

When the phloem-related EPG variables were analyzed in the 2nd hour (60–120 min) of recording significant differences between treatments were observed (Fig. 4). The number of times that aphids reached the phloem salivation phase (E1) was significantly higher on CMV-infected than on mock-inoculated plants (NWEI 60–120 min: 0.17 ± 0.08 and 0.50 ± 0.14 times for mock-inoculated and CMV-infected plants, respectively [$U = 312.00$, $P = 0.028$]). However, the duration of these phloem phase events ("E: E1 + E2 phloem activities" Fig. 4b) was shorter for aphids exposed to CMV-infected than to mock-inoculated plants (WDE 60–120 min: 13.11 ± 6.36 and 1.36 ± 0.30 min for mock-inoculated and CMV-infected plants, respectively [$F = 1.231$, $P = 0.000$]). A similar result was observed when the duration of both phloem phases ("E1" and "E2", related to phloem salivation and phloem ingestion, respectively) were analyzed separately. Aphids spent much less time on both E1 and E2 phloem phases on CMV-infected than on mock-inoculated plants (Fig. 4c) (WDI 60–120 min E1: 9.88 ± 3.00 and 2.04 ± 0.61 min for mock-inoculated and CMV-infected plants, respectively [$F = 5.910$, $P = 0.002$]; WDI 60–120 min E2: 26.13 ± 22.88 and 0.50 ± 0.10 min for mock-inoculated and CMV-infected plants, respectively [$F = 84.972$, $P = 0.021$]).

The analysis of EPG variables in the 3rd and subsequent hour-intervals revealed no significant differences between treatments except that more probes and stylet pathway phases (C) were detected in aphids probing on non-infected plants in the 6th hour and longer duration of the stylet pathway phase in non-infected plants in the 8th hour (data not shown).

The overall analysis of the data together suggests a strong effect of CMV-infected plants on aphid probing behaviour at an early stage of the aphid–plant interaction (first 15 min). Aphids exposed to CMV-infected plants made many short superficial probes and a large number of consecutive intracellular punctures (pd) before they committed into deeper stylet pathway activities towards the vascular bundle. Furthermore, CMV-infected plants had a clear impact on stylet activities of *A. gossypii* at the phloem level in the 2nd hour of recording. Aphids did not prefer CMV-infected plants as a feeding source and stayed significantly longer on sustained phloem ingestion activities on mock-inoculated than on CMV-infected plants.

4. Discussion

Plant virus transmission and spread is dependent on the virus–vector relationship, which can be modified or modulated by the virus in order to obtain an adaptive advantage (Blua and Perring, 1992). These modulations may be mediated by alterations in the plant defence mechanisms and the nutritional conditions of the plant (Blua et al., 1994). Plant viruses are known to induce specific changes in the emission of plant volatiles and alter plant physiology and morphology (Bosque-Pérez and Eigenbrode, 2011; Mauck et al., 2012). Previous studies demonstrate that both visual and olfactory cues emitted by the plant can alter aphid behaviour to discriminate between non-infected or infected plants (Ajayi and Dewar, 1983; Eigenbrode et al., 2002; Medina-Ortega et al., 2009; Mauck et al., 2010). After aphids land and start to probe, gustatory and nutritional cues present in infected plants may alter the performance and the ability of aphids to colonize and reproduce (Ajayi, 1986; Fereres et al., 1990; Blua et al., 1994).

Here, an evident plant-mediated indirect effect of CMV on the alighting, settling and probing behaviour of *A. gossypii* has been described. Our work shows that aphids that land on a CMV-infected cucumber plant had a higher propensity to migrate to mock-inoculated plants than those migrating in the opposite direction. This propensity of winged aphids to switch from CMV-infected to non-infected cucumber plants occurs at some point between 30 and 60 min after landing (Fig. 1). A similar result was found when using CMV-infected squash, which retained fewer aphids than healthy release plants both at 30 min and 24 h after release (Mauck et al., 2010). Our free-choice settling preference study also shows that winged aphids prefer to settle and reproduce in mock-inoculated than in CMV-infected cucumber plants 48 h after release (Figs. 2 and 3). Therefore, it appears that aphids do not accept CMV-infected plants as a host plant for sustained feeding soon after landing. Our EPG results are consistent with those obtained in the alighting and settling behaviour studies as shown by the reduced duration of the aphid's phloem salivation and ingestion phases on CMV-infected plants in the 2nd hour of recording. Aphids exposed to CMV-infected cucumber were able to reach the phloem sieve elements many times and even at a higher frequency than those on mock-inoculated plants but then rejected sustained phloem feeding. As consequence of its feeding rejection, aphids spent more time in pre-phloem activities when exposed to CMV-infected plants, a behaviour that eventually enhances the transmission of non-persistently transmitted viruses. Previous studies using the NP potyvirus *Zucchini yellow mosaic virus* (ZYMV, *Potyviridae: Potyvirus*) also showed that winged *A. gossypii* spent less time in phloem feeding than those in noninfected plants (Blua and Perring, 1992). It is well known that phloem feeding decreases aphid transmission of non-persistently transmitted viruses such as ZYMV (Fereres et al., 1992). These results suggest that feeding deterrence may occur at the phloem level. Possibly, NP viruses are able to induce the synthesis of chemical compounds that deter aphids from sustained phloem sap ingestion, although more studies are needed to confirm this. Similarly, aphid feeding deterrent compounds have been located in the phloem sap of melon plants carrying the *Vat* resistance gene (Chen et al., 1997).

Alternatively, the profile of volatile compounds emitted by CMV-infected plants could change after consecutive sieve element stylet punctures such that aphids no longer maintain their preference for infected plants and are pushed away to another host. It is well known that plant volatiles emitted by CMV-infected plants play a key role in aphid behaviour at the early stages of aphid–plant interaction. Mauck et al. (2010) demonstrated that plant odours emitted by CMV-infected squash plants attracted more aphids than mock-inoculated or untouched squash plants in the first hour of observations. Our findings show a similar trend as more aphids tend to settle on CMV-infected than on mock-inoculated cucumber plants in the first 30 min of observations, although this tendency was reverted in later counts (Fig. 2). Therefore, results suggest that odours emitted by CMV-infected plants attract and arrest aphids at an early stage before they start to probe. When aphids land and start probing they make many consecutive brief probes and intracellular punctures in the first 15 min. Then, a much higher frequency of brief consecutive stylet punctures in the mesophyll and phloem sieve elements is observed on CMV-infected than in mock-inoculated plants in the 60–120 min time interval (Fig. 4). It is possible that such aphid stylet activity can promote changes in the plant volatile emission profile of infected plants and that the initial plant attraction to aphids reverts into feeding deterrence. If such is the case, plant volatiles emitted by CMV-infected plants would mediate a pull–push response in their aphid vectors, a behavioural pattern that is known to be optimal for the transmission and spread of viruses transmitted in a NP manner. The term, pull–push, the same as the stimulo-deterrent strategy, refers to behaviour-modifying stimuli used to manipulate pest abundance (e.g. dead-end trap crops used to attract and kill certain insect pests) (Cook et al., 2007).

Two components of the behaviour of winged aphids are critical to enhance the spread of NP viruses: landing preference and

probing behaviour after landing (Blua and Perring, 1992). After landing on a preferred host, aphids probe sooner and longer than on a non-preferred host (Traicevski and Ward, 2002). According to our first 15 min EPG results, at the beginning of probing, the total mean duration of the probe and the time from the end of the last intracellular puncture (pd) to the end of the probe in CMV-infected plants is reduced (Table 2), which suggests that CMV-infected plants are not recognized as a preferred host by *A. gossypii*. Moreover, CMV alters aphid probing behaviour causing not only an increase in the number of short superficial probes but also a higher number of intracellular punctures (pd), a behavioural pattern which is essential for the transmission process of non-persistently transmitted viruses (Powell, 1991). In fact, Collar et al. (1997) showed that *Myzus persicae* acquired PVY more effectively, when the duration of their probes were shorter and when a higher number of intracellular stylet punctures (pd) was recorded. The same work showed that the time elapsed from the last pd to the end of the probe was a crucial variable for subsequent transmission of the virus. Aphids transmitting PVY spent less time in stylet pathway activities after the last intracellular puncture, which suggests that aphids tend to loose non-persistently-transmitted viruses quite readily when the probe is extended for a long time after virus acquisition. Our current work, showed that aphids exposed to CMV-infected plants in the first 15 min spent less time probing after the last pd was recorded. This result confirms that CMV can manipulate the behaviour of an insect vector to optimize transmission. It is important to note that all these observed behavioural differences in the probing behaviour of *A. gossypii* were restricted to the first 15 min of exposure to CMV-infected plants and was not observed in subsequent 15–30 min time intervals. As mentioned earlier, aphids exposed to acquisition access periods longer than 15 min are known to reduce the transmission efficacy of viruses transmitted in a NP manner (Sylvester, 1954).

Our probing behaviour studies revealed a temporal and transient effect of CMV-infected plants on the behaviour of *A. gossypii* since most of the differences between treatments occurred in the first 2 h of recording. The main CMV-induced changes in aphid behaviour were concentrated in the first 15 min of recording and disappeared after the 2nd hour of probing, suggesting that the main effects of CMV on the behaviour of *A. gossypii* occur at the early stages of vector–plant interactions. All together, our alighting, settling and probing behaviour studies reveal a temporal effect of CMV infection on aphid behaviour, These temporal and transient changes in vector behaviour when exposed to plants infected with a non-persistently transmitted virus have also been reported for potyviruses infecting cucurbits. Zucchini plants infected with ZYMV at a late stage (4 wks after inoculation) had similar effects on landing rates and post alighting probing behaviour of *A. gossypii* that lead to increased virus acquisition (Blua and Perring, 1992). This same work showed that winged *A. gossypii* did not prefer ZYMV-infected zucchini plants as colonization hosts in the field at a greater rate than uninfected zucchini following a temporal response similar to the one we found for CMV-infected cucumbers. Therefore, viruses transmitted in a NP manner may follow the general rule of initially attracting or pulling their vectors into infected sources and then pushing them away soon after they start probing. The observed behavioural traits suggest that viruses have adapted to their aphid vectors in a way that optimizes NP transmission and spread. This type of manipulation appears to be related with the mode of transmission followed by the virus indicating an adaptive evolution of viruses to their aphid vectors to enhance their own spread (Mauck et al., 2012).

The accuracy of predictions of simulation models describing virus epidemics depend on the validity of the assumptions made. Existing models describing NP virus transmission assume that the probability that an aphid will land and remain on an infected or non-infected plant is the same (Ruesink and Irwin, 1986; Madden et al., 2000; Thackray et al., 2004). However, our results show that virus spread in the field is far for being a result of random encounters between virus-infected plants and their aphid vectors. In fact, plants infected by NP viruses are more attractive to aphid landing but are not preferred as feeding sources soon after aphid probing. The alterations we have described in the behaviour of aphid vectors when approaching virus-infected plants will have a dramatic impact on the temporal and spatial spread of viruses transmitted in a NP manner. These results should be considered when constructing or adjusting existing simulation models to predict the epidemics of viruses transmitted in a NP manner.

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